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Relationship of Clinical and Microbiological Variables in Patients with Type 1 Diabetes Mellitus and Periodontitis

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Background: The aim of the study was to analyze how metabolic control of type 1 diabetes is related to clinical and microbiological periodontal parameters.

Material/Methods: The study involved 56 subjects aged from 19 to 50 years divided into 2 groups: healthy subjects (the H group), and diabetic (type 1 diabetes) patients with chronic untreated generalized periodontitis (the DM group). The glycosylated hemoglobin value (HbA1c) was determined using the UniCel DxC 800 SYNCHRON System (Beckman Coulter, USA), and the concentration in blood was measured by the turbidimetric immunoinhibition method. A molecular genetic assay (Micro-IDent plus, Germany) was used to detect periodontopathogenic bacteria in plaque samples. Periodontitis was confirmed by clinical and radiological examination.

Results: *Fusobacterium nucleatum, Capnocytophaga* species, and *Eikenella corrodens* were the most frequently found bacteria in dental plaque samples (77.8%, 66.7%, and 33.4%, respectively), whereas *Aggregatibacter actinomycetemcomitans* was identified 40.7% less frequently in the DM group than in the H group. The strongest relationship was observed between the presence of 2 periodontal pathogens – *F. nucleatum* and *Capnocytophaga* spp. – and poorer metabolic control in type 1 diabetes patients (HbA1c) and all clinical parameters of periodontal pathology.

Conclusions: Periodontal disease was more evident in type 1 diabetic patients, and the prevalence of periodontitis was greatly increased in subjects with poorer metabolic control.

MeSH Keywords: Chronic Periodontitis • Diabetes Mellitus • Pathology, Oral

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Background

Diabetes mellitus is a group of metabolic disorders characterized by chronic hyperglycemia with disturbances of carbohydrates, fat, and protein metabolism resulting from the defects in insulin secretion, insulin action, or both [1,2]. The disease is characterized by an increased susceptibility to infection, poor wound healing, and increased morbidity and mortality associated with disease progression [3]. The chronic hyperglycemia of diabetes is associated with long-term damage and failure of different organs: eyes, kidneys, nerves, heart, and blood vessels. The diagnosis of diabetes mellitus is made on the basis of a host of systemic and oral signs and symptoms, including gingivitis and periodontitis, recurrent oral fungal infections, and impaired wound healing. Periodontal disease (PD) is a complication of diabetes mellitus [4,5]. Many studies suggest that individuals with diabetes have at least a 2-fold increase in the severity of PD as compared with non-diabetics. A history of poorly controlled chronic PD can alter diabetic/glycemic control [6]. PD is a chronic inflammatory disorder caused by an invasion of anaerobic bacteria into periodontal tissues, including gingival connective tissue, periodontal ligament, and alveolar bone, and the resultant tooth loss impairs oral functions. Systemic bacterial and viral infections such as the common cold or influenza result in an increased systemic inflammation, which in turn increases insulin resistance and makes it difficult for patients to control blood glucose levels [7]. Several studies suggest that patients with PD – particularly those colonized with Gram-negative bacteria such as *Porphyromonas gingivalis, Tannerella forsythia,* or *Prevotella intermedia*, have higher levels of inflammatory serum markers such as C-reactive protein, interleukin-6, fibrinogen, compared to patients without periodontal disease. Similarly, there is an increase in resistance to insulin, which decreases glycemic control [8]. Studies have provided evidence that control of periodontal infection has an impact on the improvement of glycemic control, evidenced by a decreased demand for insulin and decreased glycosylated hemoglobin (HbA1c) levels [9]. The anaerobic Gram-negative bacterial species, collectively known as the red complex, produce a broad array of virulence factors that allow them to colonize subgingival sites, disturb the host defense system, invade and destroy periodontal tissue, and promote an immunodestructive host response [10].

The function of inflammatory cells, such as polymorphonuclear neutrophils, monocytes, and macrophages, is associated with diabetes [11]. Many cross-sectional studies have demonstrated hyper-reactivity of peripheral blood neutrophils in patients with diabetes, resulting in significantly increased production of pro-inflammatory cytokines and mediators. Sakallioglu et al. reported increased levels of monocyte chemoattractant protein -1 in gingival tissues of diabetic rats without periodontitis as compared to non-diabetic rats with periodontitis [12]. Many studies have demonstrated that periodontitis affected the diabetic condition in which toxins of periodontal pathogens like P-LPS and TNF-α possibly elevated insulin resistance by inhibiting glucose incorporation into smooth muscle cells.

These data indicate that periodontal pathogens influence systemic conditions. Further investigations are necessary to clarify the relationship between diabetes and periodontal disease [13].

The purpose of this study was to emphasize the relationship between diabetes mellitus 1 type and PD, and to analyze how metabolic control of diabetes is related to clinical and microbiological periodontal parameters.

Material and Methods

Subjects

The study involved 56 subjects aged from 19 to 50 years from the Hospital of the Lithuanian University of Health Sciences. Two groups were formed by assigning 28 patients to each group: healthy subjects (15 men and 13 women) (the H group), and diabetic (type 1 diabetes) (14 men and 14 women) patients with chronic untreated generalized periodontitis (the DM group). All patients in the group DM were treated with insulin for 3–8 years at the Clinic of Endocrinology, the Lithuanian University of Health Sciences. The patients showed no evidence of diabetes complications (organ failure). Written informed consent was obtained from all patients. The standard form contained the following data: name, age, sex, and medical and past dental history.

Chronic periodontitis subjects were diagnosed with chronic generalized periodontitis based on the criteria proposed in 1999 by the World Workshop for Classification of Periodontal Diseases and Conditions [14]. Each patient was examined using a mouth mirror and a Williams graduated periodontal probe under artificial light. The inclusion criterion was no less than 20 teeth. Bleeding on probing (BOP) was recorded as present or absent within 30 s after probing with the periodontal probe. The bleeding index was calculated and expressed in percentage of the total number of points examined. Pocket probing depths (PPD) were assessed in all patients at 6 sites per tooth with a periodontal probe. The most severely affected site per sextant was selected.

A microbiologic assay was performed if a bacterium was detected in at least 1 of the 3 examined sites: 84 samples of the most affected sites were collected from 28 patients with type 1 diabetes mellitus and untreated inflammatory periodontal diseases, and 84 samples were collected from 28 healthy subjects.

Oral hygiene status was evaluated by the Simplified Oral Hygiene Index (OHI-S; Green-Vermillion simplified) [15]. The

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OHI-S index consists of 2 components: plaque index (DI), and calculus index (CI). OHI-S = DI + CI.

The degree of metabolic control was evaluated on the basis of the glycosylated hemoglobin value (HbA1c), and was determined with UniCel DxC 800 SYNCHRON System (Beckman Coulter, USA); HbA1c levels were measured by applying the turbidimetric immunoinhibition method. Good metabolic control was taken to be represented by HbA1c ≤7%, while poor control was defined as HbA1c >7%.

Exclusion criteria were: a history of any periodontal destruction and any systemic disease for the H group; a history of any systemic disease other than type 1 diabetes mellitus and chronic untreated generalized periodontitis for the DM group; pregnancy or lactation; presence of any harmful habits such as smoking and alcoholism; and antibiotic therapy within 6 months before the study.

All experiments were conducted in accordance with the rules and regulations approved by the Kaunas Regional Bioethics Committee (approval No. BE-2-76). All subjects involved in this study signed the form of consent approved by the Kaunas Regional Bioethics Committee.

Microbial sampling

Supra- and subgingival plaque samples at the most-affected sites were collected with a sterile paper point. A sterile, medium-sized, absorbent paper point was gently inserted into the depth of the gingival crevice or the periodontal pocket, and was left in place for 10 s. The specimen-laden paper point was then placed in a capped vial [16]. One specimen-laden paper was immediately placed in Amies transport medium (Brescia, Italy) and sent within 2 h to the laboratory for the isolation of *Streptococcus intermedius*. The samples were inoculated directly onto 5% sheep blood agar (BBL, USA), chocolate agar (BBL, USA), and Schaedler agar plates (BBL, USA). Sheep blood and chocolate agar plates were incubated at 35°C in an atmosphere containing 5% CO_2 for 24–48 h. *S. intermedius* colonies were identified with the Gram-Positive ID kit (BBL Crystal) identification system.

The Molecular Genetic Assay was used for combined identification of additional periodontopathogenic bacterial species

The micro-IDent and micro-IDent plus (HAIN LIFESCIENCE, Germany) test systems were used. The testing was based on the DNA-STRIP technology. Test STRIPs coated with highly specific probes that are complementary to selectively amplified nucleic acid sequences were used. The combined molecular genetic identification of 5 periodontopathogenic bacteria (*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia, Treponema denticola)* and 6 periodontopathogenic bacteria (*Parvimonas micra*, *Fusobacterium nucleatum*, *Campylobacter rectus*, *Eubacterium nodatum*, *Eikenella corrodens* and *Capnocytophaga species*) was conducted. The whole procedure was divided into 3 steps: DNR isolation from subgingival samples (Geno type DNA isolation kit, Germany), a multiplex amplification with biotinylated primers, and a reverse hybridization.

Statistical analysis

Statistical analysis was performed with the IBM SPSS Statistics 21 statistical program for Windows. Continuous variables were described as mean ± standard deviation (SD). After testing for normality, parametric and nonparametric criteria, the ANOVA and Kruskall-Wallis tests were used for group comparison. A significance level of 0.05 was selected by testing statistical hypotheses. The size of the difference between the means was considered to be significant if type II error was $\beta \leq 0.2$, and type I error was $\alpha = 0.05$.

Correlations between investigated groups and biochemical data were evaluated by the canonical correlation coefficient eta. Discriminant analysis was used to determine the system of statistically significant biochemical parameters for classifying the investigated groups.

Results

The study group consisted of 28 patients diagnosed with type 1 diabetes mellitus; the subjects mean age was 37.7±10.6 years with a range of 19–50 years. The mean age of healthy subjects (n=28) was 34.8±10.7 years, with the same range of years. The 2 groups were matched for age, oral hygiene status, bleeding on probing index, and pocket probing depths. The degree of metabolic control was evaluated on the basis of the glycosylated hemoglobin value (HbA1c) (Table 1). The age was similar in the 2 groups and did not change significantly ($P>0.05$). However, significant differences ($\alpha=0.05$, $\beta<0.001$) were recorded in the BOP, PPD, OHI-S, and HbA1c of the DM group patients as compared with the H group subjects (Table 2). The gingival bleeding on probing index was 60.0±3.33 and 7.8±3.88 in the study and healthy groups, respectively. The diabetic group presented with a mean pocket probing depths of 5.33±0.83 mm, compared to 0.2±0.058 mm in healthy subjects. The OHI-S was greater in the diabetics 3.04±0.47, as compared to 0.26±0.033 in the non-diabetics. The mean HbA1c value among the diabetic patients was 9.42±1.2.

The data for the identification of bacteria when a bacterial species was found in at least 1 of the 3 assayed sites are

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Table 1. Descriptive statistics of clinical and biochemical parameters in diagnostic groups.

Variable	H group, n=28				DM group, n=28			
	Mean	SD	Min	Max	Mean	SD	Min	Max
Age	34.8*	10.7	19.0	57.0	37.7*	10.6	20.0	57.0
HbA1c	6.0	0.245	6.0	7.0	9.42	1.2	7.0	12.0
Bleeding on probing index%	7.8	3.88	3.0	13.0	60.0	3.33	56.0	64.0
Pocket probing depths (mm)	0.2	0.058	0.1	0.3	5.33	0.83	4.0	6.0
Oral hygiene index	0.26	0.033	0.15	0.30	3.04	0.47	2.10	3.80

* The mean of age did not change significantly ($P>0.05$). HbA1c – the glycosylated hemoglobin value; DM – diabetic (type 1 diabetes) with chronic untreated generalized periodontitis; H – healthy subjects.

Table 2. Differences in the means of clinical indices in study groups.

Variable	DM and H mean difference			
	Δ	%	P	β*
Bleeding on probing index %	52.3	671.9	0.000	0.000
Pocket probing depths (mm)	5.1	2561.7	0.000	0.000
Oral hygiene index	2.8	1085.8	0.000	0.000
HbA1c	3.4	56.7	0.000	0.000

* Computed using $\alpha=0.05$; HbA1c – the glycosylated hemoglobin value; DM – diabetic (type 1 diabetes) with chronic untreated generalized periodontitis; H – healthy subjects.

Table 3. Frequencies of microbes to *F. nucleatum (F.n.), E. corrodens (E.c.), P. gingivalis (P.g.), P. intermedia (P.i.), Capnocytophaga spp. (C.s.), A. actinomycetemcomitans (A.a.), S. intermedius (S.i.)* in the researched group patients (identified in at least 1 of 3 sites).

Bacteria	Group				Difference of frequencies (%)	β*	Odds ratio (95% confidence interval)		Contingency coefficient	P
	DM group, n=27		H group, n=27							
	%	Number of cases	%	Number of cases						
F.n.	**96.3**	**26**	**18.5**	**5**	**77.8**	**0.000**	**114.40**	**(12.42–1054.99)**	**0.618**	**0.000**
E.c.	51.9	14	18.5	5	33.4	0.258	4.74	(1.39–16.21)	0.330	0.021
P.g.	63.0	17	81.5	22	–18.5	–	0.39	(0.11–1.34)	0.202	0.224
P.i.	70.4	19	44.4	12	26.0	–	2.97	(0.97–9.12)	0.254	0.098
C.s.	**77.8**	**21**	**11.1**	**3**	**66.7**	**0.000**	**28.00**	**(6.22–126.06)**	**0.557**	**0.000**
A.a.	**59.3**	**16**	**100.0**	**27**	**–40.7**	**0.021**	**0.00**	**(0.00–0.33)**	**0.451**	**0.000**
S.i.	33.3	9	59.3	16	–26.0	–	0.34	(0.11–1.04)	0.252	0.101

* Computed using $\alpha=0.05$.

presented in Table 3. *F. nucleatum* was found in 96.3% of cases in group DM and in 18.5% of subjects of the H group. *F. nucleatum* was 77.8% more frequently detected in the DM group than in the H group ($\alpha=0.05$, $\beta<0.001$). The frequency of the detection of *Capnocytophaga spp.* in the DM and the H groups was 77.8% and 11.1%, respectively. *Capnocytophaga spp.* was 66.7% more frequently detected in the DM group than in the H group ($\alpha=0.05$, $\beta<0.001$). *A. actinomycetemcomitans* was found in 59.3% of patients in the DM group and in 100% of group H subjects. *A. actinomycetemcomitans* was

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Table 4. The average classification efficiency.

Observed		Predicted group		Percentage correct
		H	DM	
Group	H	22	5	81.5
	DM	1	26	96.3
Overall percentage				88.9

DM – diabetic (type 1 diabetes) with chronic untreated generalized periodontitis; H – healthy subjects.

Table 5. Coefficients of canonical correlation eta.

Bacteria	Bleeding on probing index	Pocket probing depths	Oral hygiene index	Hb A1c
F.n.	0.781	0.770	0.787	0.742
C.s.	0.663	0.681	0.725	0.627
P.g.	0.222*	0.200*	0.129*	0.034*
P.i.	0.258*	0.293	0.338	0.276
E.c.	0.310	0.358	0.378	0.336
A.a.	0.523	0.464	0.448	0.517
S.i.	0.236*	0.255*	0.265*	0.181*
Group	0.991	0.976	0.973	0.892

* The mean of canonical functions value did not change significantly ($P>0.05$). Microbes: *F. n. – F. nucleatum; E.c. – E. corrodens; P.g. – P. gingivalis; P.i. – P. intermedia; C.s. – Capnocytophaga species; A.a. – A. actinomycetemcomitans; S.i. – S. intermedius*.
Hb A1c – the mean glycosylated hemoglobin.

identified 40.7% less frequently in the DM group than in the H group ($\alpha=0.05$, $\beta<0.001$). *E. corrodens* was more frequently found in the DM group than in the H group ($P<0.05$), whereas the presence of *P. gingivalis*, *P. intermedia,* and *S. intermedius* did not differ significantly between the DM and the H groups ($P>0.05$). Contingency coefficients (CC) have shown that *F. nucleatum* and *Capnocytophaga spp.* had the greatest relationship with the study groups (CC=0.618, $P<0.01$ and CC=0.557, respectively; $P<0.01$). Table 3 shows that the presence of *F. nucleatum* increased the odds of having DM by 114.4 times (95% CI 12.42–1054.99), and the presence of *Capnocytophaga spp.* by 28.0 times (95% CI 6.22–126.06).

Logistic regression was used to predict DM according to the presence *F. nucleatum, E. corrodens, P. gingivalis, P. intermedia, Capnocytophaga spp., A. actinomycetemcomitans,* and *S. intermedius.* The Forward Conditional method converged during 1 step and selected pattern – bacteria *F. nucleatum* to optimal pattern system. The mean efficiency of the classification was 88.9%. Valid classification was achieved in 96.3% of DM patients, which indicates the sensitivity of the method, and in 81.5% of the H group patients, which indicates the specificity of the method (Table 4).

Correlations between the clinical and microbiological data of the investigated groups were evaluated by applying the canonical correlation coefficient eta. Table 5 shows the canonical correlation coefficients eta. It can be concluded that the study group had the strongest correlation in all clinical parameters – HbA1c and bacteria *F. nucleatum,* and *Capnocytophaga spp.* – whereas the presence of *P. gingivalis, S. intermedius* or the absence of clinical parameters did not change the mean values of canonical functions significantly ($P>0.05$).

Discussion

The relationship between periodontal inflammatory disease and diabetes mellitus suggests the predisposition of systemic disease to oral infection and vice versa. In the present study, periodontal disease was initiated by a subgingival infection. Anaerobic Gram-negative pathogens *A. actinomycetemcomitans, T. forsythia, P. gingivalis* and *P. intermedia, T. denticola,* and *E. corrodens* were found to be associated with the development and progression of periodontal disease [17,18]. *P. micros, F. periodontium, and C. rectus* were predominant in subgingival

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plaque samples in the periodontitis group compared to the donor group, and *E. nodatum,* and *E. corrodens* were predominant in the gingivitis group compared to the healthy group [19]. *T. forsythia and T. denticola* are most frequently found in subgingival plaque samples of type 1 diabetic patients, and these findings correlate with poorer metabolic control of diabetes [20].The results of our study show that the presence of periodontal pathogens in subgingival plaque samples was different: *F. nucleatum, Capnocytophaga* spp.*,* and *E. corrodens* were identified more frequently in the DM group than in the H group, whereas *A. actinomycetemcomitans* was detected less frequently in the DM group than in the H group.

Biofilm-related products released into the periodontal pocked include bacterial endotoxins, chemotactic peptides, and organics acids [21]. Chaushu et al. showed that *F. nucleatum* is recognized by NCR1 and NKp46 directly and that this recognition leads to the secretion of TNF-α, a central cytokine critically involved in the pathogenesis of periodontal destruction [22]. This results in further stimulation of the host response, activation of host enzymes including matrix metalloproteinases, and the release of pro-inflammatory cytokines and prostaglandin E2, among others [23]. When analyzing the pathogenicity of *T. forsythia,* the pathogenic effect of lipopolysaccharide (LPS) and bacterial DNA from *T. forsythia* were shown to induce the production of pro-inflammatory cytokines by human macrophages, with the IL-8 secretion level of LPS from *T. forsythia* being about 1.5 times the effect of LPS from *P. gingivalis* [24]. *T. forsythia* could be internalized by macrophages and stimulate intracellular receptors. The responses of macrophages upon stimulation with *T. forsythia* are complex, involving autocrine responses to the produced cytokines [25].

This cascade of events leads to eventual destruction of periodontal tissues. The responses of the host to periodontopathic microorganisms are thought to be critically important. Neutrophils (NL) are the principal cells of the host defense system and the primary protective cells against periodontal diseases [26,27]. Released granule components from infiltrating leukocytes, such as lysosomal enzymes and reactive oxygen species, which are normally intended to degrade ingested microbes, can also lead to tissue destruction and amplification of the inflammatory response.

Not only local, but also systemic factors play an important role in the development of inflammatory periodontal disease. There appears to be a relationship between the 2 processes, whereby the consequences of diabetes mellitus serve as modifiers of the expression of periodontal pathology [28]. HbA1c was considered to be the indicator of metabolic control. Persistent poor glycemic control has been associated with the incidence and progression of diabetes-related complications, including gingivitis, periodontitis, and alveolar bone loss [29]. Our findings support data from other studies showing that in the researched groups, the strongest correlation was detected between the bacteria *F. nucleatum, Capnocytophaga spp.* and HbA1c and clinical parameters (BOP, PPD, OHI-S). Several mechanisms have been proposed to explain the increased susceptibility to periodontal diseases, including alterations in host response, subgingival microflora, collagen metabolism, vascularity, gingival crevicular fluid, and heredity patterns [21]. Chronic hyperglycemia induces a pro-inflammatory state in the gingival microcirculation, characterized by an increased vascular permeability and leukocyte and endothelial cell activation. Leukocyte-induced microvascular damage may in turn contribute to periodontal tissue damage in type 1 diabetes [30]. Poorly controlled diabetes is a risk factor for PD, as confirmed in our investigation. The positive correlation between glycemic control, HbA1c levels, and the severity of PD concurred with the findings from other reported studies [31]. The reasons for poorer periodontal health among patients with poor glycemic control could be explained by the hyperglycemic static resulting in accumulation of advanced glycation end-products (AGE), as these products in turn lead to a cascade of inflammatory reactions leading to the release of inflammatory mediators like IL-1, IL-6, TNFα, and C reactive protein, thereby enhancing the periodontal breakdown process [11,32]. It has been postulated that the interaction of receptors for AGEs (RAGE) induces an oxidant stress that may be responsible for monocytic upregulation, activation of NF-κB and the subsequent expression of mRNA, and secretion of pro-inflammatory cytokines (such as TNF-α, IL-1β and IL-6) by monocytic phagocytes involved in periodontal tissue inflammation and destruction [33]. The AGE alters the permeability of the endothelium, favoring the inflammatory processes due to the increasing of adhesion molecules expression [34]. Advanced glycosylation end-product-induced oxidative stresses (glycoxidative stress) are deleterious to β-cells, leading to mitochondrial depolarization, uncoupling of oxidative phosphorylation, large-amplitude mitochondrial swelling, and cell death. Advanced glycosylation end-products might play an important role in the progressive dysfunction and loss of β-cells by increasing β-cell mitophagy and mitochondrial morphology changes. There is evidence that mitochondria are involved in the development of diabetes [35,36].

All the evidence regarding the biologic link between diabetes and periodontal disease supports the notion that diabetes and persisting hyperglycemia lead to an exaggerated immune-inflammatory response to periodontal pathogens, resulting in more rapid and severe periodontal tissue destruction [37].

Conclusions

Poorly controlled diabetes mellitus is a risk factor for periodontal disease, as our investigation confirmed. *F. nucleatum, Capnocytophaga* spp.*,* and *E. corrodens* were the most

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frequently found bacteria in dental plaque samples, and only the presence of 2 periodontal pathogens – *F. nucleatum* and *Capnocytophaga* spp. – correlated with poorer metabolic control in type 1 diabetes patients (HbA1c) and with all clinical parameters of periodontal pathology. These findings need to be confirmed in larger studies with different diagnostic sensitivity and treatment of type 1 diabetes mellitus and PD.

Statement of conflicts of interest

The authors warrant that this article is original, does not infringe upon any copyright or other proprietary right of any third party, and is not under consideration by another journal. The authors declare that they have no conflict of interest.

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